New Isolinariins C, D and E, Flavonoid Glycosides from Linaria japonica

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New Isolinariins C, D and E, Flavonoid Glycosides from *Linaria japonica*

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Three new flavonoid glycosides named isolinariins C, D and E (1–3), two known flavonoid glycosides (4, 5) and three known flavonoids (6–8) were isolated from the whole plant of *Linaria japonica*. The structures of these compounds were determined mainly by spectroscopic analyses. The bioactivities of these isolated compounds were evaluated for their inhibitory activities against human cell line A549, collagenase, and advanced glycation end product (AGE) formation. Among the isolated compounds, isolinariins C, D and E (1, 2 and 3) showed inhibition toward AGE formation (IC_{50} values of 34.8, 35.0 and 19.5 μM, respectively). And linariin (4), pectolinarin (5) and luteolin (8) were found to be active against collagenase with IC_{50} values of 79.4, 78.6 and 40.5 μM, respectively, without significant cytotoxicity at these concentrations.

Key words *Linaria japonica*; cytotoxicity; collagenase; advanced glycation end product; flavonoid glycoside

Linaria japonica Miq. (Scrophulariaceae) is a perennial herb with elliptic and fleshy leaves. The whole plant extract is used as a Japanese folk medicine due to its diuretic, purgative and laxative properties.[1)] On our reinvestigation of the same plant, collected in sandy seashore areas of Tottori Prefecture, flavonoids, phenylethanoids, iridoids and monoterpene glucosides have been isolated so far.[2)] On further investigation of the non-polar fraction, *i.e.* a mixture of hexane and ethyl acetate layers of the same plant, five new diterpenoids were isolated.[3)] The present study on the same non-polar fraction has demonstrated the presence of three new flavonoid glycosides (**1**–**3**) along with linariin (**4**), pectolinarin (**5**), pectolinarigenin (**6**), apigenin (**7**) and luteolin (**8**) (Fig. 1) through the isolation by various chromatographic techniques such as silica gel, octadecylsilyl (ODS) and HPLC. The chemical structures of these compounds were determined mainly by spectrometric analyses such as UV, IR, high resolution electrospray ionization (HR-ESI)-MS, one and two dimensional (1 and 2D)-NMR. We also report here the inhibitory activity for collagenase and advanced glycation end products (AGEs) formation of the isolated compounds along with the cytotoxicity against human cell line.

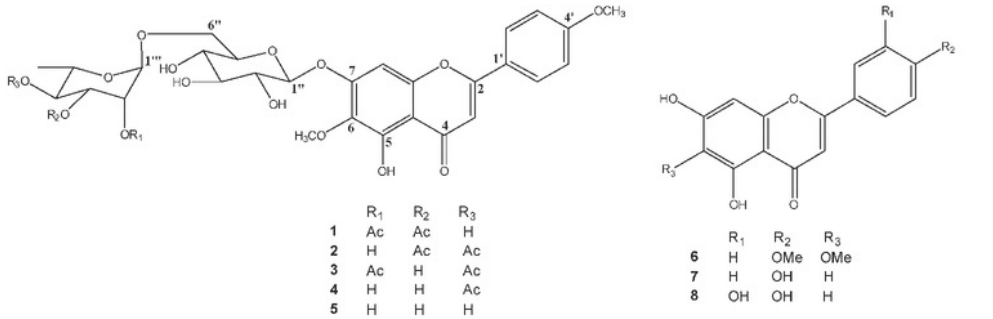
Results and Discussion

The mixture of hexane and ethyl acetate layers of a MeOH extract of *Linaria japonica* was fractionated by various types of chromatography to afford eight compounds (**1**–**8**).

Isolinariin C (**1**), $[\alpha]_D$ −4.8, was obtained as a pale yellow powder with the molecular formula $C_{33}H_{38}O_{17}$ as determined by HR-ESI-MS at *m/z* 729.1998 $[M+Na]^+$ (calcd for 729.2001). The IR spectrum indicated the presence of hydroxy (3437 cm^{-1}), ester carbonyl (1746 cm^{-1}), aromatic ring (1509 and 1460 cm^{-1}) and ether (1182 and 1054 cm^{-1}) functions.

The ^{1}H-NMR spectrum (Table 1) displayed signals due to a methyl of a rhamnose at δ_H 1.17 (d, *J*=6.2 Hz), two singlet methyl signals of acetyl groups at δ_H 1.75 and 1.93,[4)] two oxygenated methylene protons of glucose at δ_H 3.76 (m) and 4.05 (br d, *J*=9.9 Hz), a singlet signal of two methoxy groups at 3.89 (6H, s), two anomeric protons at δ_H 4.72 (br s) and 5.19 (d, *J*=7.2 Hz), two aromatic protons at δ_H 6.68 (s) and 6.79 (s), and an AA′BB′ type coupling system at δ_H 7.08 (2H, d, *J*=8.1 Hz) and 7.95 (2H, d, *J*=8.1 Hz).

The ^{13}C-NMR spectrum (Table 1) of **1** showed 31 carbon resonances that were classified by chemical shift values and heteronuclear single quantum coherence (HSQC) spectrum as; two acetyl groups (δ_C 20.6, 20.8, 171.5 and 172.1), two



	R_1	R_2	R_3
1	Ac	Ac	H
2	H	Ac	Ac
3	Ac	H	Ac
4	H	H	Ac
5	H	H	H

	R_1	R_2	R_3
6	H	OMe	OMe
7	H	OH	H
8	OH	OH	H

Fig. 1. Structures of Compounds **1**–**8**

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Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data for Compounds **1**–**3**

Position	**1**		**2**		**3**	
	δ_C	δ_H Multi (J in Hz)	δ_C	δ_H Multi (J in Hz)	δ_C	δ_H Multi (J in Hz)
2	166.9	—	166.5	—	166.9	—
3	104.5	6.68 s	104.5	6.68 s	104.6	6.70 s
4	184.6	—	184.5	—	184.6	—
5	154.4	—	154.36	—	154.4	—
6	134.0	—	134.6	—	134.7	—
7	158.0	—	157.8	—	157.8	—
8	95.5	6.89 s	95.8	6.90 s	96.1	6.95 s
9	154.6	—	154.41	—	154.5	—
10	108.0	—	107.8	—	107.9	—
1′	124.7	—	124.6	—	124.6	—
2′, 6′	129.8	7.95 d (8.1)	129.6	7.94 d (8.1)	129.7	7.97 d (8.1)
3′, 5′	115.8	7.08 d (8.1)	115.9	7.07 d (8.1)	115.9	7.09 d (8.1)
4′	164.5	—	164.6	—	164.7	—
6-OCH_3	61.7	3.89 s	61.7	3.90 s	61.7	3.90 s
4′-OCH_3	56.2	3.89 s	56.3	3.89 s	56.3	3.88 s
1″	101.6	5.19 d (7.2)	101.6	5.20 d (7.2)	101.8	5.19 d (7.2)
2″	74.8	3.58 t (8.3)	74.9	3.59 t (8.3)	75.0	3.58 t (8.3)
3″	77.9	3.52 t (8.9)	78.0	3.53 m	77.9	3.52 42 9)
4″	71.6	3.43 m	71.3	3.45 m	70.9	3.48 m
5″	77.3	3.73 m	77.2	3.73 m	76.9	3.69 m
6″	67.5	3.76 m	67.1	3.76 m	66.8	3.75 m
		4.05 brd (9.9)		4.03 brd (9.9)		3.99 m
1‴	99.3	4.72 brs	101.6	4.74 brs	98.8	4.73 brs
2‴	71.1	5.15 dd (3.3, 1.6)	70.0	3.98 m	73.9	5.06 dd (3.3, 1.6)
3‴	73.3	5.01 dd (9.9, 3.3)	73.4	5.02 dd (9.9, 3.3)	68.6	3.97 dd (9.9, 3.3)
4‴	71.3	3.42 d (9.9)	72.6	4.98 d (9.9)	75.5	4.78 d (9.9)
5‴	70.0	3.77 m	67.7	3.84 m	67.7	3.77 m
6‴	18.0	1.17 d (6.2)	17.7	0.96 d (6.2)	17.7	0.92 d (6.2)
2‴-OAc	20.6	1.93 s	—	—	20.8	2.03 s
	171.5				172.1	
3‴-OAc	20.8	1.75 s	20.8	1.82 s	—	—
	172.1		171.9			
4‴-OAc	—	—	20.9	1.96 s	21.1	2.03 s
			171.9		172.4	

Recorded at 600 MHz in CD_3OD. Chemical shifts (δ) are expressed in ppm. m: multiplet or overlapped signals.

sugars (rhamnose: δ_C 18.0, 70.0, 71.1, 71.3, 73.3 and 99.3, and glucose: 67.5, 71.6, 74.8, 77.3, 77.9 and 101.6), two methoxy carbons (δ_C 56.2 and 61.7), four sp^2 methine carbons (δ_C 95.5, 104.5, 115.8 (2×C), 129.8 (2×C)), eight sp^2 quaternary carbons (δ_C 108.0, 124.7, 134.0, 154.4, 154.6, 158.0, 164.5, 166.9) and a carbonyl carbon at δ_C 184.6. The heteronuclear multiple bond connectivity (HMBC) spectrum exhibited correlations be 9 tween δ_H 6.68 (H-3) and δ_C 124.7 (C-1′), 166.9 (C-2) and 184 9 (C-4), and between δ_H 6.89 (H-8) and δ_C 134.0 (C-6), 158.0 (C-7), 154.6 (C-9) and 108.0 (C-10) rev 37 ed the presence of a flavone skeleton (Fig. 2). Furthermore, the HMBC correlations of two methoxy groups at δ_H 3.89 (6H, s) with δ_C 134.0 (C-6) and 164.5 (C-4′), and the correlations of tw 36 nomeric protons at δ_H 4.72 (H-1‴) and 5.19 (H-1″) with 67.5 (C-6″) and 158.0 (C-7), respective 10 confirmed the linkage of these functional groups (Fig. 2). The ^{1}H- and ^{13}C-NMR spectroscopic data of **1** were closely similar to those of linariin[5,6] except for the chemical shift values of position-2‴ and 3‴ of the rhamnose moiety. The lower field 40 ted protons at δ_H 5.15 and 5.01 of **1** sugges 29 the presence of the acetyl groups on C-2‴ and C-3‴, which was confirmed by the HMBC correlations of H-2‴ (δ_H 5.15) and H-3‴ (δ_H 5.01) to the carbonyl carbon signals at 20 171.5 (2‴-OAc) and 172.1 (3‴-OAc), respectively (Fig. 2). Acid hydrolysis of **1** with 1N HCl liberated D-glucose and L-rham 35 e by HPLC analysis with optical rotation detector.[7] The coupling constant (J=7.2 Hz) of H-1″ indicated β linkage for glucose and the chemical shift values of rhamnose moiety were ind 1 tive of α-L-rhamnopyranose.[5] Based on the NMR data and acid hydrolysis, the structure of **1** was determined to be pectolinarigenin-7-*O*-(2,3-diacetyl-α-L-rhamnopyranosyl)-(1→6)-β-D-glucopyranoside. 26

Isolinariin D (**2**), $[\alpha]_D$ −8.6, was obtained as a pale yellow powder a 5 its molecular formula was determined to be $C_{33}H_{38}O_{17}$ from its positive-ion mode HR-F 22 MS data at m/z 729.1997 $[M+Na]^+$ (calcd for 729.2001). The ^{1}H- and ^{13}C-NMR spectra (Table 1) of **2** were very similar to those of **1** except for the signals of H-2‴ and H-4‴ of rhamnose, which indicated that **2** was a positional isomer of **1**. The positions 5 the two acetyl groups were deduced to be at C-3‴ and C-4‴ by analysis of the HMBC data showing correlatio 5 of H-3‴ and H-4‴ to two carbons at δ_C 171.9 (Fig. 2). The acid hydrolysis of **2** also yielded the aglycone (pectolinarigenin), D-glucose

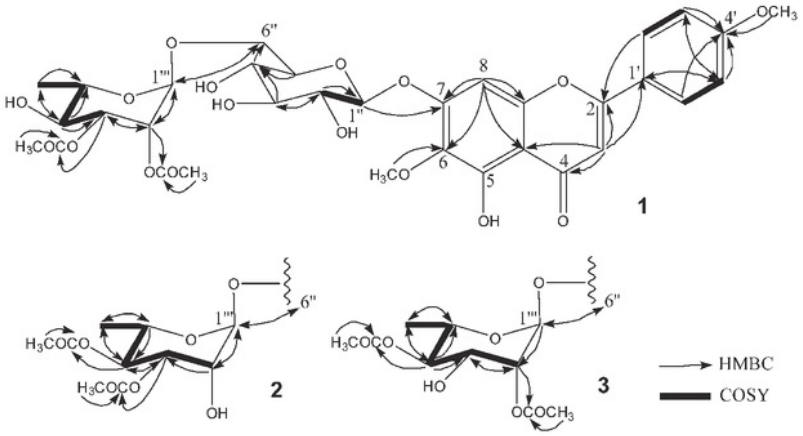

Fig. 2. HMBC and COSY Correlations of **1**–**3**

Table 2. Bioactivities of Isolated Compounds (**1**–**8**)

Isolated compounds	A549 cytotoxic (IC_{50}, μM)	AGE formation (IC_{50}, μM)	Collagenase (IC_{50}, μM)
Isolinariin C **1**	—	34.8±5.6	—
Isolinariin D **2**	—	35.0±8.8	—
Isolinariin E **3**	—	19.5±2.0	—
Linariin **4**	—	—	79.4±3.8
Pectolinarin **5**	—	—	78.6±2.4
Pectolinarigenin **6**	91.1±6.7	—	—
Apigenin **7**	—	—	—
Luteolin **8**	82.6±5.4	85.8±6.8	40.5±3.2
Aminoguanidine	n.d.	1290±31.5	n.d.
Caffeic acid	n.d.	n.d.	120±1.8
Doxorubicin	0.7±0.06	n.d.	n.d.

—: >100 μM, n.d.: not determined.

and L-rhamnose, thus the structure of **2** was determined as pectolinarigenin-7-*O*-(3,4-diacetyl-*α*-L-rhamnopyranosyl)-(1→6)-*β*-D-glucopyranoside.

Isolinariin E (**3**), $[\alpha]_D$ −6.6 ° also a pale yellow powder. The NMR data together with molecular ion at *m/z* 729.1997 $[M+Na]^+$ in HR-ESI-MS indicated that **3** was also another positional isomer of **1**. The lower field shift of H-4‴ [δ_H 4.78 (1H, d, 9.9 Hz)] and the upper field shift of H-3‴ [δ_H 3.97 (1H, dd, 9.9, 3.3 Hz)] suggested that the acetylated position was changed from C-3‴ to C-4‴ in **3**, which was further supported by a correlation between the proton at δ_H 4.78 (H-4‴) and carbon signal at δ_C 172.4 (Fig. 2) in the HMBC spectrum. The acid hydrolysis of **3** also showed the aglycone (pectolinarigenin), D-glucose and L-rhamnose. Therefore, the structure of **3** was elucidated as pectolinarigenin-7-*O*-(2,4-diacetyl-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside.

AGEs are well known to cause aging, hyperglycemia and diabetic complications. Therefore, inhibitors of AGEs formation are demanded as potential therapeutic remedy. The inhibitory effects of the isolated compounds (**1**–**8**) on AGEs formation were evaluated using a fluorescent method[8,9] (Table 2). The results showed that new compounds **1**, **2** and **3** showed stronger inhibitory activity (IC_{50}=34.8, 35.0 and 19.5 μM, respectively) than that of a reference compound, aminoguanidine (1.29 mM). Thus, the presence of acetyl groups on the rhamnose may contribute to the inhibitory activity against AGEs formation.

Collagenase is an enzyme that is known to be a member of matrix metalloproteinase (MMP) family. The agents that inhibit collagenase may have beneficial effects for maintaining healthy skin by preventing dermal matrix degradation. Therefore, the isolated compounds (**1**–**8**) were evaluated the collagenase inhibitory activity (Table 2). The results showed linariin (**4**) and pectolinarin (**5**) exhibited weak inhibition with IC_{50} values of 79.4 and 78.6 μM, respectively. Luteolin (**8**, 40.5 μM) showed stronger inhibitory activity than that of a positive control, caffeic acid (an IC_{50} value of 120 μM) as reported previously.[10]

In summary, chemical investigation of the non-polar fraction of *L. japonica* led to the isolation of eight compounds (**1**–**8**), including three new flavonoid glycosides (**1**–**3**). These isolated compounds were examined for their cytotoxicity, collagenase and AGEs formation. New compounds **1**, **2**, and **3** showed moderate inhibition of AGEs formation, and **4**, **5** and **8** for collagenase, without any cytotoxicity at the concentrations of each IC_{50} value (Table 2), which indicated that these compounds and crude extract of *L. japonica* may become an useful remedy for the AGEs associated diseases and skin deterioration.

Experimental

General ^{1}H- and ^{13}C-NMR spectra were taken on a Bruker Ultrashield 600 spectrometer at 600 MHz and 150 MHz, respectively, with tetramethylsilane (TMS) as an internal standard. IR and UV spectra were measured on Horiba FT-720 and Jasco V-520 UV/Vis spectrophotometers, respectively. Optical rotations were measured on a Jasco P-1030 digital polarimeter and a Jasco J-720 spectropolarimeter, respectively. Positive ion HR-ESI-MS was performed with an Applied Biosystems QSTAR XL NanoSpray™ system. Silica gel open column chromatography (CC) and reversed-phase [octadecyl silylated silica gel (ODS)] CC were performed on silica gel (E. Merck, Darmstadt, Germany) and Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan; Φ=35 mm, *L*=350 mm) columns, respectively. HPLC was performed on an ODS column (Inertsil ODS-3, GL Science, Tokyo, Japan; Φ=6 mm, *L*=250 mm) and the eluate was monitored with a Jasco RI-930

intelligent detector and a Jasco PU-980 intelligent pump. All reagents were purchased from TCI (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque unless otherwise specified.

Plant Material Whole plants of *Linaria japonica* were collected in late July 1990 in seashore areas of Tottori Prefecture, Japan, and a voucher specimen (90-LJ-Tottori) was deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Hiroshima University.

Extraction and Isolation The air-dried plants (2.30 kg) were extracted with MeOH (15 L) three times. The MeOH extract was concentrated to 5 L and adjusted to 95% aqueous MeOH by the addition of H_2O. This solution was then partitioned with *n*-hexane (1.5 L) two times. The remaining aqueous MeOH layer was evaporated, resuspended in 1.5 L of water, and then partitioned with ethyl acetate (1.5 L) two times and 1-butanol (1.5 L) three times successively.

The non-polar fraction (60.5 g, as a mixture of the ethyl acetate and *n*-hexane layers) was separated on a silica gel (300 g) CC with increasing polarity [hexane : $CHCl_3$ (1 : 1), 4 L, $CHCl_3$: MeOH (50 : 1, 40 : 1, 30 : 1, 20 : 1, 15 : 1, 10 : 1, 7 : 1, 5 : 1, 3 : 1, 2 : 1, MeOH, each 2 L)] yielding 12 fractions (Frs. Lj1–Lj12). Fractions Lj3 (9.61 g), Lj6 (1.07 g), Lj8 (3.43 g) and Lj10 (2.86 g) were subjected to open reversed-phase (ODS) CC in a 10% aq. MeOH (400 mL) to 100% MeOH (400 mL) linear gradient, which led to 19 fractions (Frs. Lj3-1–Lj3-19, Frs. Lj6-1–Lj6-19, Frs. Lj8-1–Lj8-19 and Frs. Lj10-1–Lj10-19, respectively). The residue of fraction Lj3-11 (62.9 mg) was recrystallized with MeOH to give pectolinarigenin (**6**, 4.0 mg). Fraction Lj6-10 (33.4 mg) was also purified by HPLC (67.5% aq. MeOH) to give luteolin (**8**, 12.1 mg). The other residue of fraction Lj6-11 (37.5 mg) was purified by preparative HPLC (45% aq. acetone) to give **7** (apigenin, 5.1 mg). Fraction Lj8-11 (238 mg) was purified by preparative HPLC (55% aq. MeOH). Three peaks appeared at 18, 25 and 35 min and were collected to give isolinariin C (**1**, 11.6 mg), isolinariin D (**2**, 18.0 mg) and isolinariin E (**3**, 5.3 mg), respectively. Then, fractions Lj10-10 (186 mg) and Lj10-11 (379 mg) were recrystallized with MeOH to give **4** (linariin, 15.5 mg) and **5** (pectolinarin, 71.1 mg), respectively.

Isolinariin C (**1**), Pale yellow powder; $[\alpha]_D^{25}$ −4.8 (c=0.77, MeOH); UV (EtOH) λ_{max} (logε) nm: 324 (3.83), 274 (3.86), 232 (3.83); IR (film) ν_{max} cm^{-1}: 3437, 2933, 1746, 1654, 1606, 1566, 1509, 1460, 1361, 1251, 1182, 1054, 837, 667; ^{1}H- and ^{13}C-NMR, see Table 1; positive HR-ESI-MS m/z 729.1998 [M+Na]$^+$ (calcd for $C_{33}H_{38}O_{17}Na$: 729.2001).

Isolinariin D (**2**), Pale yellow powder; $[\alpha]_D^{26}$ −8.6 (c=1.20, MeOH); UV (EtOH) λ_{max} (logε) nm: 322 (3.85), 272 (3.85), 233 (3.86); IR (film) ν_{max} cm^{-1}: 3443, 2932, 1735, 1653, 1607, 1462, 1510, 1458, 1360, 1250, 1182, 1044, 836, 669; ^{1}H-NMR and ^{13}C-NMR, see Table 1; positive HR-ESI-MS m/z 729.1997 [M+Na]$^+$ (calcd for $C_{33}H_{38}O_{17}Na$: 729.2001).

Isolinariin E (**3**), Pale yellow powder; $[\alpha]_D^{26}$ −6.6 (c=0.35, MeOH); UV (EtOH) λ_{max} (logε) nm: 340 (4.30), 276 (3.76), 229 (3.78); IR (film) ν_{max} cm^{-1}: 3361, 2931, 1735, 1652, 1603, 1460, 1508, 1457, 1360, 1250, 1182, 1051, 837, 670; ^{1}H-NMR and ^{13}C-NMR, see Table 1; positive HR-ESI-MS m/z 729.1997 [M+Na]$^+$ (calcd for $C_{33}H_{38}O_{17}Na$: 729.2001).

Acid Hydrolysis of 1, 2 and 3 A solution of isolinariin C (**1**), D (**2**) and E (**3**) (5 mg each) in 1 N HCl (0.2 mL) was heated at 90–100°C in a screw-capped vial for 2 h. The mixture was neutralized by addition of amberlite IRA96SB (OH^- form) and filtered. The filtrate was dried and partitioned with EtOAc–H_2O mixture (1 : 1) two times. The combined EtOAc layer was evaporated to afford an aglycone, pectolinarigenin (**6**), which was identified with NMR, MS and/or HPLC analysis with authentic sample isolated in this study. The water layer was dried *in vacuo* and dissolved in 0.2 mL of pyridine containing L-cysteine methyl ester (15 mg/mL) and reacted at 60°C for 1 h. To the mixture, a solution (0.1 mL) of *o*-toryl isothiocyanate in pyridine (5 mg/mL) was added, and it was heated at 60°C for 1 h. The final mixture was directly analyzed by HPLC [Cosmosil 5C_{18} AR II (250×4.6 mm i.d., Nacalai Tesque); 25% CH_3CN in 50 mM H_3PO_4; flow rate 0.8 mL/min; column temperature 35°C; detection 250 nm]. The t_R of the peak at 18 min coincided with that of D-glucose. The t_R of the L-rhamnose was 30 min.[7]

Cytotoxicity Assay Human lung cancer cells (A549) were obtained from Riken cell bank (RCB3677) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Japan) supplemented with 10% heat-inactivated fetal bovine serum, kanamycin (100 μg/mL) and amphotericin B (0.5 μg/mL). Into a 96-well plate, aliquots of the dimethyl sulfoxide (DMSO) solution of the test compounds (1% final concentration) were incubated with A549 cells (5×10^3 cells/well) in a CO_2 incubator at 37°C for 72 h. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added into each well and the plate was further incubated for 1.5 h. Absorbance was measured at 540 nm using a 2300 EnSpire Multimode plate reader (PerkinElmer, Inc.). DMSO was used as a negative control and doxorubicin as a positive control. The viability was compared to that of control cells incubated in the same medium without the test compounds. Measurements were performed in triplicate and the IC_{50} of the intensity of absorbance were determined graphically.[11]

AGEs Assay The reaction mixture, 10 mg/mL of bovine serum albumin (Sigma-Aldrich Japan) in 50 mM phosphate buffer (pH 7.4) containing 0.02% sodium azide, was added to a 0.5 M ribose solution. The reaction mixture was then mixed with the test compounds. After incubation at 37°C for 24 h, the fluorescent reaction products were assayed with a spectrofluorometric detector (EnSpire, PerkinElmer, Inc., Japan; Ex: 370 nm, Em: 440 nm). Measurements were performed in triplicate and the IC_{50} of the intensity of fluorescent were determined graphically.[8]

Collagenase Inhibition Assay Collagenase inhibitory activity was examined using the modified method described by Teramachi *et al.*[10] Briefly, the test compounds, 10 μg/mL of enzyme (collagenase from *Clostridium histolyticum* (Sigma-Aldrich Japan)) and 50 mM Tricine buffer (pH 7.5) were added to a 96-well microtiter plate and preincubated for 10 min at 37°C. Afterwards, the substrate solution ((7-methoxycoumarin-4-yl) acetyl-L-prolyl-L-leucylglycyl-L-leucyl-[N^{β}-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) (PEPTIDE INSTITUTE, Osaka, Japan) at a final concentration of 10 μM was added to initiate the reaction. The fluorescence values were measured at an excitation of 320 nm and an emission of 405 nm after 0 and 30 min incubation at 37°C using a fluorescence plate reader (EnSpire; PerkinElmer, Inc., Japan). These assays were performed in triplicate using caffeic acid as a positive control, and the IC_{50} of the intensity of fluorescent were determined graphically.

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Conflict of Interest The authors declare no conflict of interest.

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